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Modulation of PECAM-1 Expression and Alternative Splicing During Differentiation and Activation of Hematopoietic Cells

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Abstract PECAM-1 (CD31) is a member of immunoglobulin gene superfamily, which is highly expressed on the surface of endothelial cells and at moderate levels on hematopoietic cells. Hematopoietic cells and platelets, like endothelial cells, express multiple isoforms of PECAM-1. However, the identity and physiological role of these isoforms during hematopoiesis remains largely unknown. Here we demonstrate that PECAM-1 expression is dramatically up regulated upon phorbol myristate acetate (PMA) or transforming growth factor (TGF)-β1-mediated differentiation of leukemic HEL and U937 cells. The level of PECAM-1 expression did not significantly change during activation of Jurkat T cells by PMA or phytohaemagglutinin (PHA). Utilizing RT-PCR and DNA sequencing analysis, we show that the expression of PECAM-1 isoforms changes in a cell-type and lineage specific manner during cellular differentiation and activation. We identified a number of novel PECAM-1 isoforms previously not detected in the endothelium. These results demonstrate that regulated expression of PECAM-1 and its exonic inclusion/exclusion occurs during differentiation and/or activation of hematopoietic cells. Thus, different PECAM-1 isoforms may play important roles in generation of hematopoietic cells and their potential interactions with vascular endothelium. J. Cell. Biochem. 88: 1012–1024, 2003.

Key words: CD31; inflammation; cell–cell interactions; hematopoiesis

PECAM-1 (CD31) is a cell adhesion molecule that is highly expressed on endothelial cells and some hematopoietic cells. Among leukocytes, PECAM-1 is expressed by monocytes and neutrophils, as well as by a unique subset of T lymphocytes, particularly naive $CD8^+$ T cells. Bone marrow stem cells and transformed cell lines of the myeloid and megakaryocytic lineage also express PECAM-1. PECAM-1 is localized to tube-like endothelial structures formed in vitro or lumen-facing areas of blood vessels [Ilan et al., 1999; Sheibani and Frazier, 1999; Cao et al., 2002], and becomes diffusely distributed at the leading edge on migrating endothelial cells [Schimmenti et al., 1992]. Pretreatment of monocytes or neutrophils, as well as endothelial cells, with anti-PECAM-1 antibodies inhibited transendothelial migration of leukocytes in vitro [Albelda et al., 1991; Muller et al., 1993; Muller, 1995; Berman and Muller, 1995] and in vivo [Vaporciyan et al., 1993; Muller, 1995], indicating that PECAM-1 molecules on both endothelial cells and leukocytes contribute to the transmigration process [Ilan et al., 2000]. However, the role of PECAM-1 and its isoforms in hematopoiesis and transendothelial migration remain largely unknown.

PECAM-1 plays an important role in the leukocyte and endothelial cell adhesion cascades. This may involve both homophilic [Muller et al., 1989; Albelda et al., 1990; Newman et al., 1990;

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Sun et al., 1996; Newton et al., 1997] and heterophilic [Albelda et al., 1991; Muller et al., 1992; DeLisser et al., 1993; Piali et al., 1995; Buckley et al., 1996] interactions of PECAM-1 on EC and hematopoietic cells. These adhesions may be modulated, at least in part, by alternative splicing of PECAM-1 cytoplasmic domain. The alternative splicing of exon 14 in murine PECAM-1 alters the binding characteristics of PECAM-1 when expressed in L cells [Yan et al., 1995]. We have recently demonstrated that expression of different PECAM-1 isoforms in epithelial (MDCK) cells differentially modulates the ability of these cells to form cadherin-mediated cell–cell adhesion [Sheibani et al., 2000]. This is perhaps mediated through differential interactions of PECAM-1 isoforms cytoplasmic domain with intracellular proteins [Jackson et al., 1997; Hua et al., 1998; Pellegatta et al., 1998; Pumphrey et al., 1999; Ilan et al., 1999, 2000]. Therefore, the cytoplasmic domain of the PECAM-1 isoforms may actively participate in modulating cell-cell interactions and differentiation.

PECAM-1 plays an important role in hematopoiesis [Watt et al., 1993] and its expression is regulated during differentiation or activation of hematopoietic cells [Zehnder et al., 1992; Watt et al., 1993; Goldberger et al., 1994a]. During early hematopoietic development PECAM-1 is highly expressed on CD34 enriched human hematopoietic progenitor cells and its expression is greatly reduced in more mature stages of all lineages that lack CD34 [Watt et al., 1993]. PECAM-1 expression can also be regulated during cell activation. Activation of granulocytes by FMLP [Stockinger et al., 1990], and activation of human T cells by PHA [Zehnder et al., 1992] leads to down-regulation of PECAM-1 molecule expression. In contrast, in monocytes stimulated with FMLP, PMA, IFN-γ, or LPS PECAM-1 expression is not affected [Stockinger et al., 1990; Goldberger et al., 1994a]. PECAM-1 appears to play a negative regulatory role in T cell receptor-mediated signal transduction [Newton-Nash and Newman, 1999]. We have recently shown that hematopoietic cells, like endothelial cells, express multiple isoforms of PECAM-1 in a species and lineage-specific manner [Wang et al., 2002; Wang and Sheibani, 2002]. However, the identity and the role of PECAM-1 isoforms during differentiation or activation of hematopoietic cells require further investigation.

HEL and U937 cell lines provide a useful model system to study megakaryocytic and/or macrophage differentiation [Koren et al., 1979; Papayannopoulou et al., 1983; Molla and Block, 2000] and have been used to identify a number of transcription factors involved in hematopoiesis [Clarke and Gordon, 1998; Rooney and Calame, 2001]. We have utilized HEL and U937 cell lines to study the lineage specific modulation of PECAM-1 expression and its alternative splicing during differentiation. These cells were induced to differentiate down specific myelomonocytic pathways by PMA [Goldberger et al., 1994a], erythroid differentiation by TGF-β1 (U937) [Lastres et al., 1994; Bergh et al., 1997], and T cell activation by PMA or PHA (Jurkat) [Gillis and Watson, 1980; Weiss et al., 1984; Zehnder et al., 1992; Sebzda et al., 2002]. We demonstrate that both PECAM-1 expression and its alternative splicing are modulated during differentiation and activation of the lymphocytic cells. Our results demonstrate that PECAM-1 undergoes alternative splicing generating a number of novel isoforms during differentiation and/or activation of hematopoietic cells. Thus, PECAM-1 isoforms with different adhesive properties may play a role in hematopoiesis and inflammation.

MATERIALS AND METHODS

Cell Lines

The human erythroleukemia HEL, human macrophage U937 and human T lymphocyte (lymphoblast) Jurkat cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 with 10% heat inactivated fetal bovine serum and 1 mM sodium pyrovate.

Differentiation and Activation of Human Hematopoietic Cells

The differentiation induction of HEL and U937 cells was carried out according to Goldberger et al. [1994a]. Briefly, HEL and U937 cells were induced to differentiate down the megakaryocytic (HEL) or the macrophage (U937) lineages in the presence of 20 nM PMA (phorbol myristate acetate, Calbiochem, San Diego, CA) for different lengths of time. The U937 cells were also incubated with 10 ng/ml TGF-β1 (R&D, Minneapolis, MN) for 1 day to promote erythroid differentiation [Bergh et al., 1997]. Jurkat cells were activated by incubation

with PMA (20 nM) for 4 days or PHA (1 μg/10^6 cells, Sigma, St. Louis, MO) for 1 day [Zehnder et al., 1992]. All the treatments were performed in growth medium and DMSO was used for solvent control.

Northern Blot Analysis

Poly A^+ RNA was isolated from various cell lines as described previously [Sheibani et al., 1991]. Poly A^+ RNA (5 μg) was size fractionated in a 1.2% agarose formaldehyde gel and transferred to zeta-probe membrane (Bio-Rad, Hercules, CA), prehybridized and hybridized to random primer ^{32}P-labeled full-length human cDNA probes for PECAM-1 (a gift of Dr. Peter J. Newman, Blood Research Center of Southeastern Wisconsin, Milwaukee, WI). The blot was also probed with a cDNA for GAPDH to control for loading. Northern blots were scanned using a PhosphorImager Storm 860 (Molecular Dynamics). Quantitative analysis of PECAM-1 and GAPDH expression (loading control) was performed using ImageQuant 5.2 software (Molecular Dynamics). Relative levels of PECAM-1 expression compared to GAPDH were determined by comparison of the band intensities of PECAM-1 with GAPDH.

Western Blot Analysis

Approximately 10^7 cells were centrifuged at 300*g* for 5 min, gently washed with cold TBS (20 mM Tris, 150 mM NaCl, pH 7.4) twice, and lysed in 0.5 ml of lysis buffer (20 mM Tris, pH 7.4, 2 mM EDTA, 1% Triton X-100, and protease inhibitors cocktail, Roche Biochemicals, Indianapolis, IN) with a brief sonication. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (25 μg) was analyzed by SDS–PAGE (4–20% Tris-Glycine Gel, Invitrogen, Grand Island, NY), transferred to Nitrocellulose, and blotted with either an antibody that reacts with the extracellular domain of human PECAM-1 (recognizes all PECAM-1 isoforms; SEW 16, a gift of Dr. Peter Newman) or an antibody that reacts with exon 14 of murine PECAM-1 (recognizes PECAM-1 isoforms with exon 14; Sheibani et al., 1999]. Following incubation with appropriate secondary antibody, blot was washed, and developed using ECL (Amersham, Piscataway, NY). Same blot was probed with both antibodies.

Identification of Alternatively Spliced PECAM-1 Isoforms

The mRNAs (prepared above) were utilized as template for RT-PCR analysis (Superscript™ One-Step RT-PCR, Gibco-BRL, Gaithersberg, MD) to amplify the cytoplasmic domain of all possible PECAM-1 isoforms. The sense primer was designed as 5′-atggatcc^{2021}AGG AAA GCC AAG GCC AGG^{2038}-3′, which spans the border of exon 9 and 10 within the intracellular domain. The anti-sense primer was designed as 5′-cggaattc ^{2371}CCT TGC TGT CTA AGT CCT^{2354}-3′, which spans the border of exon 16 and 3′-untranslated region. The primers carry a BamHI and an EcoRI recognition sequence (lowercase letters) to facilitate subsequent cloning of PCR products. PCR products were examined on a 2.4% agarose gel to assess their integrity and expected size. For cloning, PCR products were directly purified by using Qiagen PCR Purification Kit (Qiagen, Valencia, CA), digested with BamHI and EcoRI, cleaned with the same kit, ligated into the pGEX-2T vector (Pharmacia, City, Code) cut with same enzymes, and transformed into *E. coli* DH5α. Bacterial colonies were screened by BamHI and EcoRI digestion of DNA minipreps and those with inserts were sequenced using the Big Dye (University of Wisconsin Biotechnology Center). Identification of PECAM-1 isoforms was performed as described previously [Sheibani et al., 1999; Wang and Sheibani, 2002].

RESULTS

PECAM-1 Expression During Differentiation and Activation of Hematopoietic Cells

PECAM-1 is an early marker of endothelial and hematopoietic cells that can be detected in precursor cells. However, the role of PECAM-1 isoforms in hematopoiesis requires further delineation. In vitro differentiation of myeloid leukemic cell lines and activation of T cells are useful models to examine the role of PECAM-1 during these processes [Zehnder et al., 1992; Goldberger et al., 1994a]. We have examined changes in PECAM-1 expression and alternative splicing utilizing HEL cells as a model for the megakaryocyte/platelet lineage differentiation, the monoblastic cell line U937 cells as a model for the monocytic/macrophage lineage differentiation, and the lymphoblast Jurkat cell line as a model for T cell activation.

PECAM-1 mRNA expression in the HEL, U937 and Jurkat T cells following incubation with PMA, PHA, TGF-β1, or solvent control is shown in Figure 1 (upper panel). The relative expression of PECAM-1 compared to GAPDH (as a loading control) is shown in the lower panel. In untreated cells, PECAM-1 was expressed to varying degrees. The highest expression was in untreated HEL cells (Fig. 1). PECAM-1 expression was significantly up regulated in U937 cells in the presence of PMA or TGF-β1. PECAM-1 expression was also up regulated in PMA treated HEL cells, compared to control. However, the relative increase in PECAM-1 becomes less prominent because of a significant increase in GAPDH mRNA (loading control, Fig. 1). PECAM-1 expression was increased 5-fold in U937 cells incubated with PMA for 2 days, and 1.7-fold by 4 days, when compared to DMSO control. Incubation of U937 cells with TGF-β1 resulted in 2.5-fold increase in PECAM-1 expression. PMA or PHA did not significantly affect PECAM-1 expression in Jurkat cells.

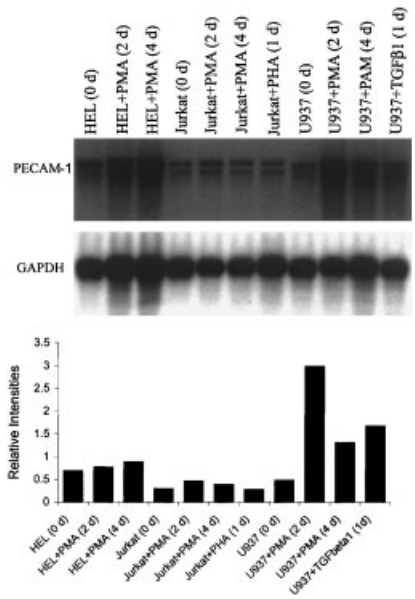

Fig. 1. Northern blot analysis of RNA isolated from control (0 day) or cells incubated with PMA, PHA, or TGF-β1 for designated time points. Approximately, 5 μg of poly A^+ RNA was separated on a 1.2% agarose formaldehyde gel, transferred to zeta-prob membrane, prehybridized, and hybridized to random primer ^{32}P-labeled full-length human PECAM-1 cDNA. The blot was also probed with a cDNA for GAPDH to control for loading. Please note increased expression of PECAM-1 mRNA in cells incubated with PMA or TGF-β1. The relative amounts of PECAM-1 compared to GAPDH, are shown in the bottom panel.

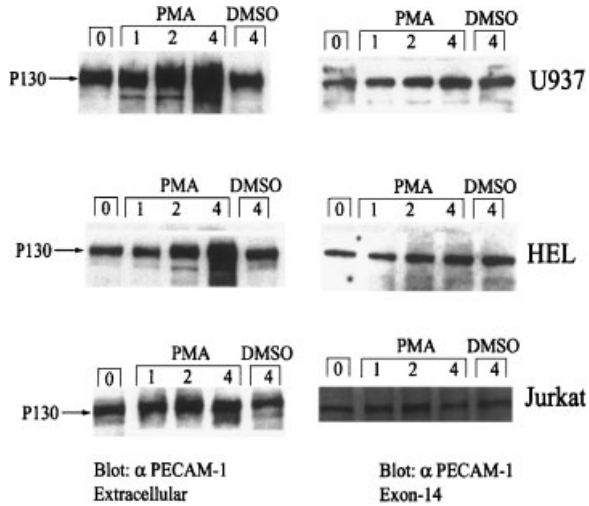

Fig. 2. Western blot analysis of PECAM-1 in hematopoietic cells. Lysates were prepared from control (DMSO) or cells incubated with PMA for designated time points as described in Materials and Methods. Equal amounts of protein (25 μg) from each sample were analyzed under reducing conditions utilizing SDS–PAGE. Proteins were transferred to nitrocellulose membrane and blotted with the rabbit polyclonal antibody to extracellular domain (**left panel**) or antibody to exon 14 (**right panel**) of PECAM-1. Please note the presence of a lower molecular weight band in cells incubated with PMA. This experiment was repeated three times with identical results.

The changes in PECAM-1 expression levels were confirmed by Western blot analysis (Fig. 2). The expression pattern of PECAM-1 isoforms in U937 cells during monocytic/macrophage differentiation was examined. U937 cells were incubated with PMA or DMSO (control) and protein lysates were prepared for Western blot analysis. PECAM-1 is a 130-kDa protein with approximately 40% of its molecular mass being contributed by carbohydrate residues [Newman et al., 1990]. PECAM-1 expression was significantly up regulated following incubation with PMA (1, 2, 4 days). Incubation of U937 cells with PMA for 2 or 4-days dramatically increased PECAM-1 expression, compared to untreated or DMSO (solvent control) treated cells. We observed an additional faster migrating protein band after 1 day, which became more prominent after 4 days of incubation with PMA. The PECAM-1 antibody, which reacts with the extracellular domain of human PECAM-1, recognized the full-length PECAM-1 (130 kDa) in U937 cells incubated with DMSO. However, an additional lower molecular weight band (approximately 110–120 kDa) was detected in cells incubated with PMA (Fig. 2, left panel). To

confirm that the additional band on immunoblot is the product of alternatively spliced PECAM-1 isoform(s), the same blot was probed with an antibody to murine PECAM-1 exon 14 (recognizes isoforms with exon 14; Sheibani et al., 1999] (Fig. 2, right panel). The exon 14 antibody only detected the band corresponding to the full-length PECAM-1 in cells incubated with PMA or DMSO. These data suggest that the lower molecular weight band is the product of PECAM-1 isoform(s) lacking exon 14. We observed similar results during differentiation of HEL cells (Fig. 2). These are consistent with the increased percentages of the isoforms without exon 14 in cells incubated with PMA (Tables I and II). Jurkat cells incubated with PMA did express similar levels of PECAM-1 compared to control cells (Fig. 2), consistent with the Northern blot data (Fig. 1). Furthermore, we did not observe significant amounts of the lower molecular weight protein band in the treated Jurkat cells using the antibody that reacts with the extracellular domain of PEACM-1. A similar pattern was observed when the same blot was probed with exon 14 antibody. This is consistent with the detection of a lower percentage of isoforms without exon 14 in these cells (Table III).

Distribution of the PECAM-1 Isoforms During Differentiation and Activation of Hematopoietic Cells

To determine whether the multiple bands that were seen in Northern (Fig. 1) and Western (Fig. 2) blots are the result of alternative splicing of PECAM-1 mRNA, we examined the pattern of PECAM-1 isoforms during differentiation of HEL (Fig. 3), and U937 (Fig. 4) cells, or activation of Jurkat (Fig. 5) cells. RT-PCR analysis of RNA was performed utilizing primers designed to expand the entire PECAM-1 cytoplasmic domain to allow amplification of all PECAM-1 isoforms. The largest molecular weight band, corresponding to full-length PECAM-1 cytoplasmic domain, is ~350 bp. The lower band(s) are considered to be the alternatively spliced PECAM-1 isoform(s). The RT-PCR analysis indicated the presence of multiple bands in proliferating HEL cells. They correspond to a size of ~350 and ~300 bp. In contrast a single band, corresponding to the expected size (~350 bp) of full-length PECAM-1 cytoplasmic domain was detected in the proliferating U937 or resting Jurkat cells. An additional band (~160 bp), only detected in the control HEL cells, is a contaminating band corresponding to a gene with unknown product (NCBI: AB02694 or AAH01069) which shares 94% nucleotide sequence homology with PECAM-1 in the primer sequences.

The RT-PCR analysis demonstrated multiple bands during differentiation or activation of hematopoietic cells. Incubation with PMA resulted in changes in the RT-PCR banding pattern of HEL cells (Fig. 3). At least four bands (~350, 300, 260, and 190 bp, respectively) were visible in samples prepared from HEL cells incubated with PMA for 2 and 4 days. The U937

TABLE I. Distribution of PECAM-1 Isoforms During Differentiation of HEL Cells

PECAM-1 isoforms	HEL + PMA, 0 day (28)	HEL + PMA, 2 days (34)	HEL + PMA, 4 days (28)
Full*	33	3	29
Δ 11, 12, & 14	ND	6	ND
Δ 11, 12, & 14	ND	3	ND
Δ 12	ND	ND	4
Δ 12, & 13	ND	15	7
Δ 12, 13, & 14	ND	18	7
Δ 12, 13, 14, & 15	ND	3	7
Δ 12 & 14	ND	29	11
Δ 12, 14, & 15	ND	3	ND
Δ 13	7	ND	ND
Δ 13 & 14	7	9	4
Δ 13 & 15	ND	3	ND
Δ 14	30	6	32
Δ 14 & 15	4	3	ND
Δ 15	19	ND	ND

Isoforms of PECAM-1 were identified by cloning and sequencing the RT-PCR products from mRNA isolated from untreated or PMA treated HEL cells as described in Materials and Methods. The numbers in parentheses indicate the total number of PECAM-1 clones examined. ND, not detected.
*The number indicates the frequency in percent at which each isoforms was detected.

TABLE II. Distribution of PECAM-1 Isoforms During Differentiation of U937 Cells

PECAM-1 isoforms	Full*	Δ13	Δ13 & 14	Δ14	Δ14 & 15	Δ15
U937 + PMA, 0 day (27)	67	ND	4	22	ND	7
U937 + PMA, 2 days (28)	32	4	4	39	7	14
U937 + PMA, 4 days (24)	75	ND	ND	25	ND	ND
U937 + TGF-β1, 1 day (28)	93	ND	ND	ND	ND	7

Isoforms of PECAM-1 were identified by cloning and sequencing the RT-PCR products from mRNA isolated from untreated, PMA, or TGF-β1 treated U937 cells as described in Materials and Methods. The numbers in parentheses indicate the total number of PECAM-1 clones examined. ND, not detected.
*The number indicates the frequency in percent at which each isoforms was detected.

cells incubated with PMA generated a lower molecular weight band (~300 bp), but TGF-β1 incubation did not result in any obvious changes in the RT-PCR pattern (Fig. 4). The PMA-treated Jurkat cells only generated a lower molecular weight band (~300 bp) after 4 days. No obvious changes can be detected in the PHA-(1 day) or PMA-treated (2 days) Jurkat cells (Fig. 5). Taken together, the RT-PCR results indicated that production of alternatively spliced PECAM-1 isoforms occurs in the cells incubated with PMA.

Identification of PECAM-1 Isoforms During Differentiation and Activation of Hematopoietic Cells

The identity of the PECAM-1 isoforms was confirmed by cloning and sequencing of the RT-PCR products as described in the Materials and Methods. Tables I, II, and III demonstrate the PECAM-1 isoforms and frequency at which they were detected in the human hematopoietic cells during their differentiation or activation.

Table I shows the distribution of PECAM-1 isoforms in HEL cells during their incubation with PMA. The proliferating HEL cells expressed six PECAM-1 isoforms. These included the full-length and isoforms that lack exons 13, 13&14, 14, 14&15, and 15. The full-length (33%) and Δ14 (30%) PECAM-1 were the predominant isoforms detected. HEL cells expressed a number of novel PECAM-1 isoforms which were only present in cells incubated with PMA. These included the Δ11,12,&13, Δ11,12,&14, Δ12, Δ12&13, Δ12,13,&14, Δ12,13,14,&15, Δ12&14, Δ12,14,&15, and Δ13&15 PECAM-1. Thus, a specific expression pattern of PECAM-1 isoforms occurs during megakaryocytic differentiation of HEL cells. The full-length PECAM-1 becomes a minor percentage of PECAM-1 isoforms (3%), while the Δ12&14 (29%) is the predominant isoform detected after 2 days of PMA treatment. The full-length (29%) and Δ14 (32%) PECAM-1 become the predominant isoforms after 4 days of PMA treatment. However, the percentage of isoforms without exon 14 increases from 41 to 80% after 2 days and 61% after 4 days of incubation with PMA. The isoform Δ15 was not detected during differentiation of HEL cells. The distribution of PECAM-1 isoforms in the HEL cells is in agreement with the RT-PCR pattern (Fig. 3).

The exclusion of exon 11 from PECAM-1 cytoplasmic domain (Δ11,12,&13 and Δ11,12, &14) has not been previously reported. This results in a shift in the reading frame shortening the cytoplasmic tail of PECAM-1 by 40 (in

TABLE III. Distribution of PECAM-1 Isoforms During Activation of Jurkat Cells

PECAM-1 isoforms	Full*	Δ11, 12, & 13	Δ12	Δ12 & 13	Δ12 & 15	Δ13	Δ14 & 15	Δ15
Jurkat, 0 day (25)	76	ND	4	ND	ND	ND	12	8
Jurkat + PHA, 1 day (22)	82	ND	ND	5	ND	5	5	5
Jurkat + PMA, 2 days (24)	92	ND	ND	ND	ND	ND	4	4
Jurkat + PMA, 4 days (22)	27	5	9	ND	5	14	41	5

Isoforms of PECAM-1 were identified by cloning and sequencing the RT-PCR products from mRNA isolated from untreated, PMA, or PHA treated Jurkat T cells as described in Materials and Methods. The numbers in parentheses indicate the total number of PECAM-1 clones examined. ND, not detected.
*The number indicates the frequency in percent at which each isoforms was detected.

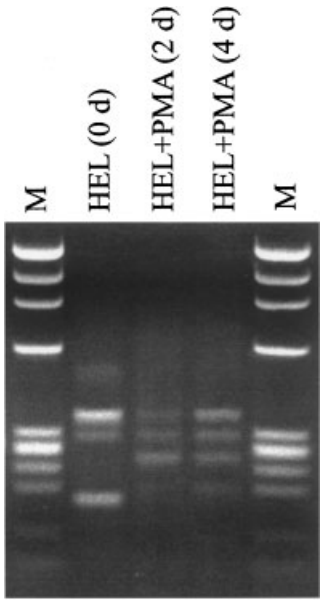

Fig. 3. The RT-PCR analysis of PECAM-1 isoforms in HEL cells. The RT-PCR products were amplified from HEL cells mRNA isolated from control or cells incubated with PMA for designated time points as described in Materials and Methods. The PCR products were separated on a 2.4% agarose gel, stained with ethidium bromide, and photographed. M designates the molecular weight marker and the bands correspond to 1350, 1078, 872, 603, 310, 280, 234, 194, and 72 bp, respectively. Please note the presence of multiple DNA bands in HEL cells which change upon incubation with PMA.

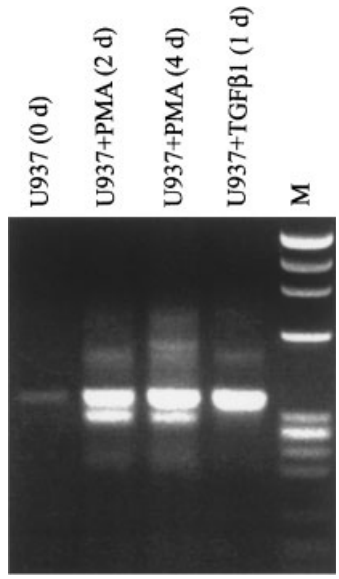

Fig. 4. The RT-PCR analysis of PECAM-1 isoforms in U937 cells. The RT-PCR products were amplified from the U937 mRNA isolated from control or cells incubated with PMA or TGF-β1 for designated times as described in Materials and Methods. The PCR products were separated on a 2.4% agarose gel, stained with ethidium bromide, and photographed. M designates the molecular weight marker and the bands correspond to 1350, 1078, 872, 603, 310, 280, 234, 194, and 72 bp, respectively. Please note the presence of a faster migrating band in cells incubated with PMA but not TGF-β1.

Δ11,12,&13) and 24 (in Δ11,12,&14) amino acids, respectively, shifting the termination codon upstream (Figs. 6 and 7). The differentiating HEL cells generated PECAM-1 isoforms lacking exon 12, 13, and 14 with higher frequencies. HEL cells incubated with PMA for 2 days produced more PECAM-1 isoforms compared to 4 days of PMA. In addition, HEL cells in general produced a greater number of PECAM-1 isoforms than U937 (Table II) and Jurkat (Table III) cells. The PECAM-1 isoforms detected in the differentiating HEL cells included exon 15, while it was excluded from the isoforms detected in proliferating HEL cells.

Table II shows the distribution of PECAM-1 isoforms in the proliferating and differentiating U937 cells. The proliferating U937 cells generated four different PECAM-1 isoforms. These included the full-length and Δ13&14, Δ14, and Δ15 isoforms. The full-length (67%) and Δ14 (22%) were the predominant isoforms. The U937 cells produced two new PECAM-1 isoforms, which were only detected during incubation

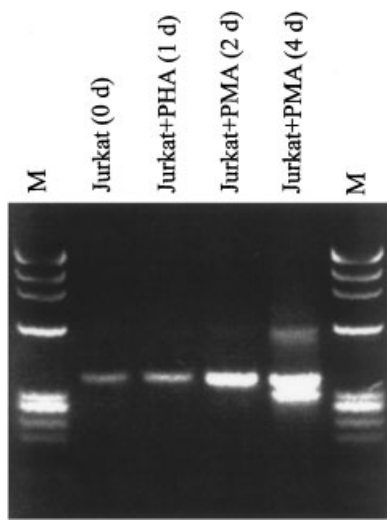

Fig. 5. The RT-PCR analysis of PECAM-1 isoforms in Jurkat cells. The RT-PCR products were amplified from the mRNA isolated from control or cells incubated with PHA or PMA for designated times as described in the Materials and Methods. The PCR products were separated on a 2.4% agarose gel, stained with ethidium bromide, and photographed. M designates the molecular weight marker and the bands correspond to 1350, 1078, 872, 603, 310, 280, 234, 194, and 72 bp, respectively. Please note the presence of a faster migrating band in cells incubated with PMA for 4 days.

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Full           AGG AAA GCC AAG GCC AAG CAG ATG CCA GTG GAA ATG TCC AGG CCA GCA GTA CCA CTT CTG AAC TCC
Δ11,12&13      AGG AAA GCC AAG GCC AAG CAG ATG CCA GTG GAA ATG TCC AG- --- --- --- --- --- --- --- ---
Δ11,12&14      AGG AAA GCC AAG GCC AAG CAG ATG CCA GTG GAA ATG TCC AG- --- --- --- --- --- --- --- ---
Δ12&13         AGG AAA GCC AAG GCC AAG CAG ATG CCA GTG GAA ATG TCC AGG CCA GCA GTA CCA CTT CTG AAC TCC
Δ12,13&14      AGG AAA GCC AAG GCC AAG CAG ATG CCA GTG GAA ATG TCC AGG CCA GCA GTA CCA CTT CTG AAC TCC
Δ12,13,14&15   AGG AAA GCC AAG GCC AAG CAG ATG CCA GTG GAA ATG TCC AGG CCA GCA GTA CCA CTT CTG AAC TCC
Δ12&14         AGG AAA GCC AAG GCC AAG CAG ATG CCA GTG GAA ATG TCC AGG CCA GCA GTA CCA CTT CTG AAC TCC
Δ12,14&15      AGG AAA GCC AAG GCC AAG CAG ATG CCA GTG GAA ATG TCC AGG CCA GCA GTA CCA CTT CTG AAC TCC
Δ12&15         AGG AAA GCC AAG GCC AAG CAG ATG CCA GTG GAA ATG TCC AGG CCA GCA GTA CCA CTT CTG AAC TCC
Δ13&15         AGG AAA GCC AAG GCC AAG CAG ATG CCA GTG GAA ATG TCC AGG CCA GCA GTA CCA CTT CTG AAC TCC

AAC AAC GAG AAA ATG TCA GAT CCC AAT ATG GAA GCT AAC AGT CAT TAC GGT CAC AAT GAC GAT GTC AGA AAC CAT
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AAC AAC GAG AAA ATG TCA GAT CCC AAT ATG GAA GCT AAC AGT CAT TAC G-- --- --- --- --- --- --- --- ---
AAC AAC GAG AAA ATG TCA GAT CCC AAT ATG GAA GCT AAC AGT CAT TAC G-- --- --- --- --- --- --- --- ---
AAC AAC GAG AAA ATG TCA GAT CCC AAT ATG GAA GCT AAC AGT CAT TAC G-- --- --- --- --- --- --- --- ---
AAC AAC GAG AAA ATG TCA GAT CCC AAT ATG GAA GCT AAC AGT CAT TAC G-- --- --- --- --- --- --- --- ---
AAC AAC GAG AAA ATG TCA GAT CCC AAT ATG GAA GCT AAC AGT CAT TAC G-- --- --- --- --- --- --- --- ---
AAC AAC GAG AAA ATG TCA GAT CCC AAT ATG GAA GCT AAC AGT CAT TAC G-- --- --- --- --- --- --- --- ---
AAC AAC GAG AAA ATG TCA GAT CCC AAT ATG GAA GCT AAC AGT CAT TAC GGT CAC AAT GAC GAT GTC GGA AAC CAT

GCA ATG AAA CCA ATA AAT GAT AAT AAA GAG CCT CTG AAC TCA GAC GTG CAG TAC ACG GAA GTT CAA GTG TCC TCA
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--- --- --- --- --- --- --- --- --- -AG CCT CTG AAC TCA GAC GTG CAG TAC ACG GAA GTT CAA GTG TCC TCA
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--- --- --- --- --- --- --- --- --- -AG CCT CTG AAC TCA GAC GTG CAG TAC ACG GAA GTT CAA GTG TCC TCA
--- --- --- --- --- --- --- --- --- -AG CCT CTG AAC TCA GAC GTG CAG TAC ACG GAA GTT CAA GTG TCC TCA
--- --- --- --- --- --- --- --- --- -AG CCT CTG AAC TCA GAC GTG CAG TAC ACG GAA GTT CAA GTG TCC TCA
GCA ATG AAA CCA ATA AAT GAT AAT AAA G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

GCT GAG TCT CAC AAA GAT CTA GGA AAG AAG GAC ACA GAG ACA GTG TAC AGT GAA GTC CGG AAA GCT GTC CCT GAT
--- --- --- --- --- -AT CTA GGA AAG AAG GAC ACA GAG ACA GTG TAC AGT GAA GTC CGG AAA GCT GTC CCT GAT
GCT GAG TCT CAC AAA G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -AT
--- --- --- --- --- -AT CTA GGA AAG AAG GAC ACA GAG ACA GTG TAC AGT GAA GTC CGG AAA GCT GTC CCT GAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -AT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
GCT GAG TCT CAC AAA G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -AT
GCT GAG TCT CAC AAA G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
GCT GAG TCT CAC AAA GAT CTA GGA AAG AAG GAC ACA GAG ACA GTG TAC AGT GAA GTC CGG AAA GCT GTC CCT G--
--- --- --- --- --- -AT CTA GGA AAG AAG GAC ACA GAG ACA GTG TAC AGT GAA GTC CGG AAA GCT GTC CCT G--

GCC GTG GAA AGC AGA TAC TCT AGA ACG GAA GGC TCC CTT GAT GGA ACT TAG ACA GCA AGG
GCC GTG GAA AGC AGA TAC TCT AGA ACG GAA GGC TCC CTT GAT GGA ACT TAG ACA GCA AGG
GCC GTG GAA AGC AGA TAC TCT AGA ACG GAA GGC TCC CTT GAT GGA ACT TAG ACA GCA AGG
GCC GTG GAA AGC AGA TAC TCT AGA ACG GAA GGC TCC CTT GAT GGA ACT TAG ACA GCA AGG
GCC GTG GAA AGC AGA TAC TCT AGA ACG GAA GGC TCC CTT GAT GGA ACT TAG ACA GCA AGG
--- --- --- --- --- --- --- AGA ACG GAA GGC TCC CTT GAT GGA ACT TAG ACA GCA AGG
GCC GTG GAA AGC AGA TAC TCT AGA ACG GAA GGC TCC CTT GAT GGA ACT TAG ACA GCA AGG
--- --- --- --- --- --- --- AGA ACG GAA GGC TCC CTT GAT GGA ACT TAG ACA GCA AGG
--- --- --- --- --- --- --- AGA ACG GAA GGC TCC CTT GAT GGA ACT TAG ACA GCA AGG
--- --- --- --- --- --- --- AGA ACG GAA GGC TCC CTT GAT GGA ACT TAG ACA GCA AGG
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Fig. 6. The cDNA sequences of the cytoplasmic domain of the novel PECAM-1 isoforms. The nucleotide sequence encoding the cytoplasmic domain of the isoforms Δ11,12&13, Δ11,12&14, Δ12&13, Δ12,13&14, Δ12,13,14&15, Δ12&14, Δ12,14&15, Δ12&15, and Δ13&15 is compared to the sequence for full-length PECAM-1 isoform. The deleted sequences are indicated by hyphens (-). The predicated exon sequences of human PECAM-1 were adapted from Newman et al. [1990].

with PMA. These include Δ13 and Δ14&15 PECAM-1 isoforms. The full-length PECAM-1 was detected at high frequency (32%) after 2 days, while the Δ14 (39%) was the predominant isoform. After 4 days, only two PECAM-1 isoforms, the full-length (75%) and Δ15 (25%) were detected. However, the percentage of isoforms without exon 14 increased from 26–50% after 2 days of incubation with PMA and then declined to 25% after 4 days.

The U937 cells incubated with TGF-β1 expressed two PECAM-1 isoforms, the full-length (93%) and Δ15 (7%). This pattern is very similar to that detected in human primary T cell and platelets (Wang and Sheibani, 2002). Thus, TGF-β1 results in inclusion of exons generating

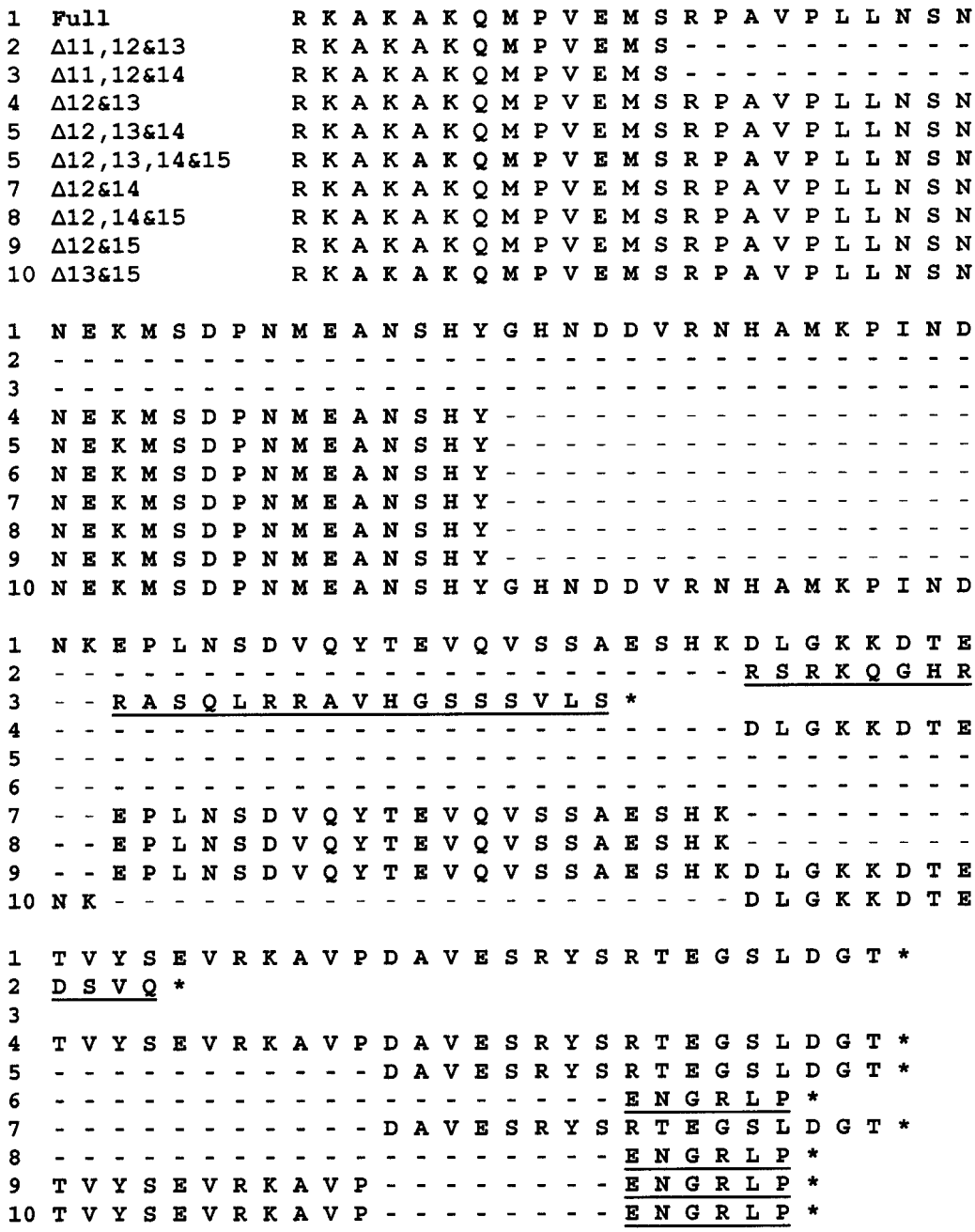

Fig. 7. The amino acid sequences of the cytoplasmic domain of the novel PECAM-1 isoforms. The amino acid sequence of the human PECAM-1 isoforms Δ11,12,&13, Δ11,12,&14, Δ12&13, Δ12,13,&14, Δ12,13,14,&15, Δ12&14, Δ12,14,&15, Δ12&15, and Δ13&15 is compared to the sequence for full-length PECAM-1 isoform. The deleted sequences are indicated by hyphens (-). The putative exon sequences of human PECAM-1 were indicated according to the cDNA sequence data (above). The underlined sequences indicate changes in the reading-frame of the amino acid sequence.

a higher number of the full-length PECAM-1 isoform. In contract, PMA results in exclusion of exons generating a higher number of alternatively spliced PECAM-1 isoforms.

Table III shows the distribution of PECAM-1 isoforms in control and during activation of Jurkat cells. The resting Jurkat cells expressed four different PECAM-1 isoforms. These included the full-length, Δ12, Δ14&15, and Δ15 PECAM-1. The full-length (76%) and Δ14 (12%) were the predominant isoforms. The incubation of Jurkat cells with PMA or PHA produced four PECAM-1 isoforms that were only present in the activated cells. These include Δ11,12,&13,

Δ12&13, Δ12&15, and Δ13 PECAM-1 isoforms. The full-length PECAM-1 was the predominant isoform in Jurkat cells incubated with PHA or PMA for 1 day or 4 days. The Δ14 PECAM-1 isoform (41%) was the predominant isoform detected in Jurkat cells incubated with PMA for 4 days. Thus, PMA treatment resulted in exclusion of exon 11, 12, 13, 14, and 15 in the Jurkat cells and Δ14 PECAM-1, but not the full-length PECAM-1, is the predominant isoform. The production of Δ11,12,&13, Δ12&15, and Δ13 are strongly induced by PMA at 4 days (Table III). The Δ12 PECAM-1 isoform was also detected in cells incubated with PMA for 4 days. However, the percentage of isoforms without exon 14 does not increase in these cells until 4 days after incubation with PMA. The incubation with PHA resulted in exclusion of exon 13, and generated two isoforms Δ13 and Δ12&13. However, the predominant isoform in the PHA treated cells was the full-length PECAM-1 (82%).

Nucleotide and Amino Acid Sequences of the Novel PECAM-1 Isoforms

The presence of various PECAM-1 isoforms has been characterized in human endothelium [Wang et al., 2002] and hematopoietic cells and platelets [Wang and Sheibani, 2002]. These include the full-length PECAM-1 and isoforms Δ12, Δ13, Δ14, Δ15, Δ13&14, and Δ14&15. The Δ13 and Δ13&14 isoforms were only detected in human endothelium. Nine new PECAM-1 isoforms were detected during differentiation or activation of lymphocytic cells. These included Δ11,12,&13, Δ11,12,&14, Δ12&13, Δ12,13,&14, Δ12,13,14,&15, Δ12&14, Δ12,14,&15, Δ12&15, Δ13&15 isoforms. The cDNA and amino acid sequences of all the novel PECAM-1 isoforms detected in this study are shown in Figures 6 and 7, respectively. This systematic investigation of the alternatively spliced PECAM-1 isoforms verified the putative alternative splicing sites of human PECAM-1 mRNA molecules previously indicated by Newman et al. [1990] and Kirschbaum et al. [1994]. Therefore, PECAM-1 undergoes alternative splicing during differentiation or activation of hematopoietic cells generating a number of isoforms with perhaps different adhesive properties.

DISCUSSION

PECAM-1 plays an important role in endothelial cell–cell interactions during angiogenesis [DeLisser et al., 1997; Sheibani et al., 1997; Sheibani and Frazier, 1998, 1999; Cao et al., 2002], and leukocyte-EC interaction during transendothelial migration [Muller et al., 1993; Muller, 1995; Wakelin et al., 1996; Nakada et al., 2000]. Expression of PECAM-1 in hematopoietic and endothelial precursor cells and its modulation during hematopoiesis, both in vitro and in vivo, suggests an important role for PECAM-1 in development of hematopoietic cells and their interactions with endothelium [Watt et al., 1993; Goldberger et al., 1994a]. We have recently demonstrated that multiple isoforms of PECAM-1 are expressed in the endothelium [Sheibani et al., 1999; Wang et al., 2002] and hematopoietic cells and platelets [Wang and Sheibani, 2002], whose expression in the endothelium is developmentally regulated [Sheibani et al., 1999]. However, the role of PECAM-1 and its isoforms in hematopoiesis remain largely unknown. Here, we demonstrate that: (1) PECAM-1 expression levels is regulated during differentiation and activation of human lymphocytic cells; (2) human lymphocytic cells expressed alternatively spliced multiple PECAM-1 isoforms whose expression pattern is regulated during differentiation and activation in a cell-type and lineage specific manner; (3) nine novel PECAM-1 isoforms were detected during differentiation or activation, which are not previously detected in the endothelium; and (4) the product of different PECAM-1 isoforms is translated in hematopoietic cells. Therefore, the regulation of PECAM-1 expression and its exonic inclusion/exclusion by alternative splicing may play important roles during hematopoiesis and inflammation.

During early hematopoietic development PECAM-1 is highly expressed on CD34 enriched human hematopoietic progenitor cells and its expression is greatly reduced in more mature stages of all lineages that lack CD34. The expression of PECAM-1 is then up regulated during terminal myeloid differentiation [Watt et al., 1993]. Here, we have utilized an in vitro cell differentiation model to assess PECAM-1 expression and alternative splicing during hematopoietic cell differentiation. PMA-induced monocytic/macrophage (U937) differentiation resulted in a 5-fold increased in PECAM-1 expression, while TGF-β1 treatment resulted in a 2.5 fold increase (Fig. 1). PECAM-1 expression was also significantly up regulated in PMA treated HEL cells. This is consistent with the

previous report by Goldberger et al. [1994a]. HEL and U937 cells also expressed different PECAM-1 isoforms upon incubation with PMA and TGF-β1 (Tables I and II). We did not detect a significant change in PECAM-1 expression during incubation of Jurkat cells with PHA or PMA at RNA (Fig. 1) or protein (Fig. 2) levels. This is in contrast to the early report by Zehnder et al. [1992] who showed decreased expression of PECAM-1 in Jurkat cells incubated with PHA. Thus, up-regulation of PECAM-1 expression may not be necessary for T cells activation. However, our results show that the alternative splicing of PECAM-1 is modulated during this process (Table III) generating a number of isoforms. Utilizing antibodies, which react with the extracellular or intracellular (exon 14) domains of PECAM-1 we demonstrate that not only PECAM-1 isoforms are generated during differentiation and activation of lymphocytic cells but also they get translated (Fig. 2). There appears to be a delay in the translation of PECAM-1 RNA. PECAM-1 isoforms as detected by RT-PCR were present after 2 days of incubation with PMA in U937 and HEL cells concomitant with increased PECAM-1 mRNA expression. However, the increased protein levels became more prominent after 4 days (Figs. 1–4; Tables I and II). The Jurkat cells expressed a higher number of PECAM-1 isoforms after 4 days of incubation with PMA. This may explain, at least in part, the failure to detect PECAM-1 isoform products that lack exon 14 in these cells (Figs. 2 and 5; Table III). TGF-β1 incubation of U937 cells resulted mainly in inclusion of exons in PECAM-1 RNA (Table II), thus, generating a similar protein-banding pattern to the control cells (not shown). These results demonstrate that the product of different PECAM-1 isoforms are made during differentiation or activation of lymphocytic cells and strongly argue against the possibility that the multiple bands detected in the immunoblots are different glycosylation variants of PECAM-1 as has been previously suggested [Goldberger et al., 1994a,b]. Therefore, hematopoietic cells may exhibit different adhesive properties by modulating the expression and alternative splicing of PECAM-1. However, the role of different PECAM-1 isoforms and their adhesive properties in hematopoietic cells awaits further investigation.

PECAM-1 undergoes alternative splicing generating a number of isoforms in hematopoietic cells [Wang and Sheibani, 2002]. Changes in the expression of PECAM-1 levels and the pattern of PECAM-1 isoforms may play a role during hematopoiesis. Here we show that lymphocytic cells express multiple isoforms of PECAM-1 whose pattern of expression changes during differentiation or activation (Tables I, II, and III; Wang and Sheibani, 2002]. Several novel PECAM-1 isoforms were identified during cell differentiation or activation (Tables I, II, and III). There were nine PECAM-1 isoforms produced during megakaryocytic differentiation (Table I), two isoforms during macrophage differentiation (Table II), and four isoforms induced during activation of T cells (Table III). The expression of PECAM-1 isoforms occurred in a differentiation/activation and cell-type/lineage specific manner. The differentiating HEL and U937 cells expressed multiple PECAM-1 isoforms much earlier during differentiation than the activated T cells (Tables I, II, and III). Thus, expression of PECAM-1 isoforms may be regulated during cell differentiation or T-cell activation. Differentiation specific expression of PECAM-1 isoforms may provide a mechanism that can modulate the adhesive function of PECAM-1during hematopoiesis. In the differentiating cells, a greater number of PECAM-1 molecules lack exon 14 (80% at 2 days and 61% at 4 days in HEL cells, and 50% at 2 days and 25% at 4 days in U937 cells; Tables I and II). In the activated Jurkat cells, 41% of PECAM-1 isoforms lack exon 14 only after 4 days of incubation with PMA (Table III). The exon 14 is recognized as a modulator of PECAM-1 adhesive properties [Yan et al., 1995; Famiglietti et al., 1997; Sheibani et al., 2000]. The exclusion of exon 14 may promote PECAM-1 mediated homophilic interaction during cellular aggregation or transendothelial migration of hematopoietic cells. Therefore, PECAM-1 isoform switching may provide a mechanism by which PECAM-1 adhesive properties can be regulated.

In summary, our results show that regulated expression of PECAM-1 isoforms occurs during differentiation or activation of blood cells, suggesting that alternative splicing of PECAM-1 mRNA is involved in regulating its function during hematopoiesis or inflammation. Further characterization of these alternatively spliced PECAM-1 isoforms and the identities of the cellular signaling molecule(s) that specifically interact with these isoforms will allow us to

elucidate the role of PECAM-1 in hematopoiesis and inflammation.

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